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Kinetic analysis of regulatory events in G_1 leading to proliferation or quiescence of Swiss 3T3 cells

(G_1 arrest/G_0 state/epidermal growth factor/platelet-derived growth factor/insulin)

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ABSTRACT **Kinetic analysis of cellular response to serum deprivation or inhibition of protein synthesis was performed on Swiss 3T3 cells. Time-lapse cinematographic analysis of individual cells transiently exposed to serum-free medium (with or without the addition of purified growth factors) or cycloheximide enabled a detailed mapping of the magnitude and variability of cellular response in different parts of the cell cycle. In all cells, in all stages of the cell cycle, serum deprivation resulted in inhibition of protein synthesis, but only in postmitotic cells in the first 3–4 hr of G_1 (here denoted the G_1pm phase) did it produce cell-cycle arrest. During G_1pm, the cells are highly dependent on the continuous presence of serum growth factors and a high level of protein synthesis in order to progress toward mitosis. A 1-hr exposure to serum-free medium or to cycloheximide was sufficient to force most G_1pm cells into a state of quiescence (G_0), from which the cells required 8 hr to return to G_1pm. During G_1pm the cells complete the growth factor-dependent processes leading to commitment for proliferation. Thereafter they enter the growth factor-independent pre-DNA-synthetic part of G_1 (here denoted G_1ps). The commitment process in G_1pm could be successfully completed in the presence of platelet-derived growth factor as the only supplied growth factor. Epidermal growth factor and insulin were insufficient for the completion of the commitment processes in G_1pm, although they were able to temporarily prevent the G_1pm cells from entering G_0 during serum starvation. Under conditions optimal for proliferation, the cells complete the commitment processes in G_1pm within a remarkably constant time period. Almost all cells in the population left G_1pm and entered G_1ps between the third and fourth hour after mitosis. The duration of G_1ps, on the other hand, showed a large intercellular variability consistent with a transition-probability event. In fact, G_1ps accounts for most of the variability in G_1 and cell cycle time.**

When cells in culture experience environmental conditions that are suboptimal for proliferation, the cellular response varies depending on cell type. Nontransformed cells usually cease to proliferate in a cell cycle-specific fashion. They are arrested in the G_1 phase or enter a state of quiescence (G_0) from G_1 after deprivation of serum growth factors (1–6) or nutrients (7, 8) or after cell crowding (density-dependent inhibition) occurs in the culture (9, 10). In contrast, many chemically or virally transformed cells and cells of tumor origin often respond differently to suboptimal culture conditions. Although cell proliferation gradually slows, the cells do not readily enter G_0 but instead remain in the cell cycle, which they traverse slowly until most cells eventually die as a result of the environmental conditions (11–15).

The ability of nontransformed cells as opposed to the inability (or reduced ability) of transformed cells to enter a specific G_0 state in response to changed environmental conditions most likely reflects one of the fundamental growth-control mechanisms that is operating stringently in normal cells but is improperly relaxed in tumor cells. Future studies of the molecular basis of these growth control events in G_1 would be facilitated if such studies could focus on a defined and very limited stage within G_1 that is of particular importance for the specific G_0 arrest. To search for such a stage and map its precise location within G_1 would therefore seem meaningful.

Our aim was to make a detailed kinetic analysis of the efficiency by which G_0 arrest was induced among individual G_1 cells located at different positions between mitosis and S-phase. Such detailed and quantitative mapping of G_1 was made possible simply by performing time-lapse cinematographic analysis of cells exposed to brief periods of serum deprivation or cycloheximide-induced inhibition of protein synthesis.

MATERIALS AND METHODS

Cell Culture. Mouse embryo fibroblasts (Swiss 3T3, purchased from Flow Laboratories) were maintained in monolayer cultures in tissue culture bottles. The stock cultures were grown in humidified 5% CO_2 in Dulbecco's modified Eagle's (DME) medium (16) supplemented with 10% (vol/vol) fetal calf serum (FCS), 50 units of penicillin per ml, and 50 μg of streptomycin per ml. The cells were removed from the bottles for transfer by treatment with 0.25% (wt/vol) trypsin in Tris-buffered saline containing 0.5 mM EDTA. The line was maintained by seeding 50,000 cells per 25-cm^2 tissue culture bottle and transferring them every 4–5 days. The cells were never allowed to reach confluence. Cells used for experimental purpose were grown in 25-cm^2 tissue culture bottles or in plastic Petri dishes, some of which contained a glass coverslip at the bottom. The cell density at the beginning of each experiment was 5–6 $\times$ 10^3 cells per cm^2. Insulin was obtained from Vitrum (Stockholm, Sweden). Epidermal growth factor (EGF) (17) was purchased from Collaborative Research. Platelet-derived growth factor (PDGF) (18, 19) was kindly given to us by B. Westermark, Å. Wasteson, and Carl-Henrik Heldin (the Wallenberg Laboratory, Uppsala, Sweden).

Time-Lapse Cinematography. A 10-ml tissue culture bottle containing exponentially growing cells, previously stored in an incubator where it had equilibrated in 5% CO_2, was carefully sealed and then placed in an inverted microscope with an attached video-camera system for time-lapse cinematography. The temperature of the medium was kept at 37°C.

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; DME, Dulbecco's modified Eagle's (medium); FCS, fetal calf serum.

In the starvation experiments, the serum-containing medium was removed and the cells were briefly rinsed in DME medium. The cells were exposed for 0.25–8 hr to serum-free DME medium or to DME medium supplemented with FCS and various concentrations of cycloheximide (Sigma), equilibrated in 5% CO_2 as described above. After the experimental period was terminated, DME medium (preequilibrated with 5% CO_2) supplemented with 10% FCS was added. Cell ages (time elapsed from the last mitosis) at the onset of the experimental period and following intermitotic times were determined by subsequent analyses of the video recordings. Cells were analyzed for 24–36 hr before the experimental period of serum starvation and most of the cells (>90%) had an established cell age at the beginning of the experimental period.

Autoradiography. Protein and DNA syntheses in cells growing on glass coverslips were estimated from incorporation of [^{3}H]leucine (20 μCi/ml of medium; New England Nuclear, 50 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]thymidine (1 μCi/ml; New England Nuclear, 25 Ci/mmol) into acid-precipitable material. At the end of each experiment, the cultures were briefly washed twice in 0.9% (wt/vol) NaCl solution, fixed in 95% (vol/vol) ethanol for 1–24 hr, and thereafter maintained in an air-dried state until autoradiography was performed. Before the film (Kodak AR-10 stripping film) was applied, cells were treated in 5% (wt/vol) trichloroacetic acid at 4°C for 5 min and then washed in cold running water for 20 min to remove unincorporated [^{3}H]leucine. After an exposure period of 7–10 days, the autoradiographs were developed with Kodak D19 developer (3.5 min at 18°C), fixed in Kodak acid x-ray fixative with hardener (5 min at 18°C), washed in cold running water for 20 min, dried, and stained in hematoxylin and eosin. The percentage of [^{3}H]thymidine-labeled cells and number of silver grains per cell ([^{3}H]leucine) were determined with a light microscope.

RESULTS

The progression of 3T3 cells through different parts of the cell cycle in the presence or absence of serum growth factors was studied by time-lapse cinematography. Cells growing in DME medium with 10% FCS were exposed to serum-free DME medium for 15 min to 8 hr in different experiments. After the serum-starvation periods were terminated, the cells were again exposed to DME medium containing 10% FCS for an additional 48 hr. The time intervals to reach mitosis for the individual cells that were in different parts of the cell cycle (i.e., had different cell ages) during the period of serum starvation were determined from the time-lapse recordings. Results from three different experiments, in which serum was absent for 0.5, 1.0, or 8.0 hr, are presented in Fig. 1. Postmitotic cells younger than 3 hr were temporarily arrested in the cell cycle for many hours (intermitotic delay) as a result of the serum starvation, whereas cells older than 4 hr were unaffected by the serum starvation and underwent mitosis on schedule. The cells thus lost their serum dependence in a rapid and synchronous fashion between the third and fourth hour after mitosis (Fig. 3). The response to serum starvation among the young postmitotic cells was very rapid. As can be seen in Fig. 1, about half of these postmitotic cells responded by delayed mitosis after 30 min in serum-free medium (Fig. 1*A*), and virtually all young postmitotic cells responded to 1 hr in serum-free medium (Fig. 1*B*). Even after exposure to serum-free medium for as little as 15 min, a significant proportion of young postmitotic cells (on the average 28% in three different experiments, data not shown) were efficiently arrested in the cell cycle for many hours.

The magnitude of the intermitotic delay (i.e., the time for which postmitotic cells younger than 3 hr were arrested in the cell cycle) was calculated for different periods of serum starvation (1.0, 2.0, 4.0, and 8.0 hr). This was done by subtracting 15.5 hr (average intermitotic time of control cells, Fig. 3) from the actual intermitotic times recorded after the different periods of serum starvation (Fig. 1).

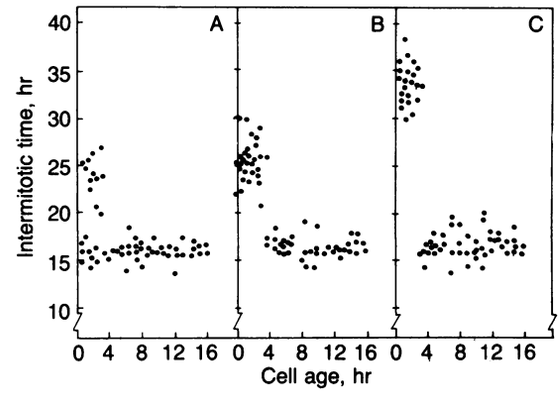

FIG. 1. Relation between cell age (time elapsed after mitosis) at the beginning of serum starvation and intermitotic time. Exponentially growing cells at a density of 6000 cells per cm^2 were exposed to serum-free DME medium for 0.5 (*A*), 1.0 (*B*), or 8.0 hr (*C*), whereupon they were then exposed to DME medium supplemented with 10% FCS for 48 hr. The cell ages and intermitotic times were determined by time-lapse cinematography.

As shown in Fig. 2, a time as short as 1 hr in serum-free medium is sufficient to delay the onset of the subsequent mitosis by as much as 9 hr (8 hr more than the actual period of serum starvation). After 2, 4, or 8 hr in serum-free medium, intermitotic delays of 10, 12, or 16 hr, respectively, were found. Thus, in all these situations the onset of mitosis was delayed by the actual time in serum-free medium plus an additional 8 hr. The latter probably represents an initial setback to G_0, from which the cells require 8 hr to reenter G_1 upon readdition of serum. This would imply that the whole intermitotic delay takes place in G_1. This conclusion is supported by autoradiographic data of time-lapse-analyzed cells, showing that G_1, which is normally 7 hr long (Fig. 3), is prolonged by 9 hr (to 16 hr) in cells exposed to serum-free medium for 1 hr (data not shown, same method used as described for the determination of G_1 in Fig. 3).

Fig. 3 shows the fraction of cells in the 3T3 population that has progressed to different cell cycle stages as a function of cell age (time elapsed after mitosis). The curve at right shows that the median intermitotic time is 15.5 hr; 50% of the cells

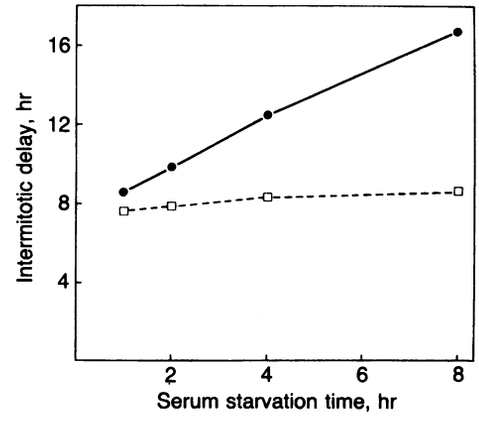

FIG. 2. Relation between serum starvation time and intermitotic delay of cells younger than 3 hr. ●, Total intermitotic delay, which includes the starvation time; □, the additional intermitotic delay (total delay minus the actual starvation time).

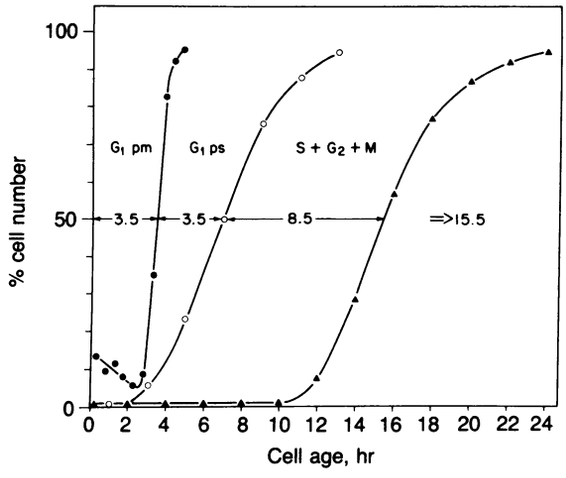

FIG. 3. Cell age distribution for different cycle phases. Curve at left (-●-) shows the cell age distribution for the transition from the serum-sensitive phase (G_1pm) to the serum-insensitive phase (G_1ps). Curve in center (-○-) shows the cell age distribution for the transition from G_1ps to S. Curve at right (-▲-) denotes the cell age distribution of entrance into the mitotic phase (M) (see text). The curves are based on analysis of 100–300 cells in five different experiments.

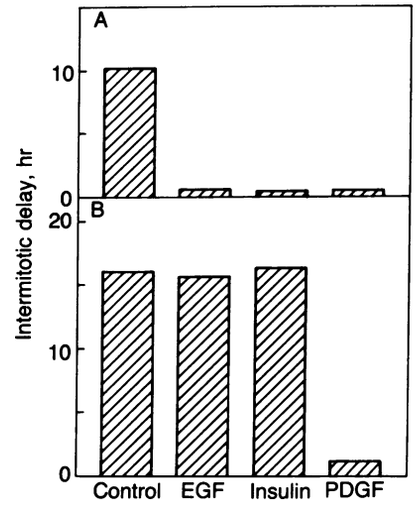

FIG. 4. The effect of addition of growth factors on the total intermitotic delay following short serum starvation of G_1pm cells (cell age <3 hr). Exponentially growing cells at a density of 6000 cells per cm² were exposed to serum-free medium (Control) or to serum-free medium plus EGF (25 ng/ml), insulin (100 μg/ml), or PDGF (25 ng/ml) for 2 (*A*) or 8 hr (*B*). Thereafter they were reexposed to medium containing 10% FCS for 48 hr. The cell age and intermitotic times were determined by time-lapse cinematography.

had undergone mitosis at the cell age of 15.5 hr (data derived from proliferating control populations). Similarly, the middle curve shows that the average length of G_1 is 7.0 hr, since 50% of the cells had entered S at the cell age of 7.0 hr. This data was obtained by determining the fraction of [^{3}H]thymidine-labeled cells in proliferating control populations of known age: Cells analyzed by time-lapse cinematography were exposed to [^{3}H]thymidine (5 μCi/ml) for 15 min, whereupon cells were fixed, photographed, and subsequently prepared for autoradiography. Cells of known age on the photograph were identified on the autoradiographs and the percentage of labeled cells was determined. The labeling index of 10–20 cells in each 2-hr age interval was determined. The curve to the left in Fig. 3 shows how the number of serum-independent cells (cells not responding by intermitotic delay after a brief exposure to serum-free medium) changes as a function of cell age. Up to 3 hr after mitosis, <10% of the cells are serum independent. Thereafter a rapid change occurs in the cells, resulting in more than 90% serum-independent cells only 1 hr later (4 hr after mitosis). The G_1 phase can thus be divided into one serum-dependent postmitotic phase (G_1pm) of a relatively constant duration of 3–4 hr (average 3.5 hr), followed by a serum-independent pre-DNA-synthetic phase (G_1ps). As judged from Fig. 3, G_1ps accounts for most of the variability in the duration of G_1 and of the whole cell cycle as well.

To study whether purified EGF, insulin, or PDGF could counteract the intermitotic delay of G_1pm cells caused by brief serum starvation, each of these growth factors was added to the serum-free medium during the serum-starvation period and was removed when the serum-containing medium was added. The results are illustrated in Fig. 4. The intermitotic delay resulting from serum starvation for 2 hr was efficiently prevented by each of the three growth factors (Fig. 4*A*). However, when the serum-starvation period was prolonged (8 hr), only PDGF efficiently counteracted the intermitotic delay (Fig. 4*B*). Neither EGF nor insulin (even at very high doses, data not shown) exerted any counteractive effect on the intermitotic delay that followed prolonged serum starvation (Fig. 4*B*).

The effect of different doses of the protein synthesis inhibitor cycloheximide on progression of cells through the cell cycle is illustrated in Fig. 5. A 2-hr exposure to cycloheximide at 10 ng/ml had a clear effect on young postmitotic cells (G_1pm cells). Although this low dose only reduces protein synthesis by 25% (data not shown), it was sufficient to induce a mitotic arrest in ≈30% of the G_1pm cells. Cycloheximide at 100 ng/ml, which reduces the level of protein synthesis to the same extent as serum starvation (≈50%, Fig. 6), induced mitotic arrest in virtually all G_1pm cells. The progression of G_1ps cells as well as S- and G_2-phase cells appeared to be unaffected, as these cells underwent mitosis on schedule. Cycloheximide at 1 μg/ml, which inhibits protein synthesis ≈90% (data not shown), blocked progression throughout all cell cycle stages.

The data on progression through the cell cycle was compared further to data on protein synthesis. The magnitude and kinetics of protein synthesis inhibition induced by serum starvation or cycloheximide was analyzed at different stages of the cell cycle. Exponentially growing control cells exposed to serum-free medium or to cycloheximide (100 ng/ml) for up to 4 hr were incubated with [^{3}H]leucine for 30 min prior to fixation. It has been shown elsewhere that the extra- and intracellular leucine pools equilibrate within a few minutes (20–22). Cells analyzed by time-lapse cinematography were processed for autoradiography, and rate of protein synthesis was determined from grain counts over individual cells. Four cell-age intervals, as defined by time elapsed since previous mitosis, were analyzed: 0–4, 4–8, 8–12, and >12 hr (Fig. 6). Both serum starvation and cycloheximide (100 ng/ml) inhibited protein synthesis by about 50% in all cell cycle stages. Maximal inhibition was seen within 2 hr after removal of serum or within 1 hr after addition of cycloheximide, irrespective of position in the cell cycle. The normal rate of protein synthesis was restored within 0.5–1 hr after removal of cycloheximide or addition of serum; this recovery was equally fast in all stages of the cell cycle.

DISCUSSION

We have analyzed the kinetics of cellular response to absence of growth factors or to inhibition of protein synthesis. The use of time-lapse cinematography in combination with brief exposure to serum-free culture medium or to cycloheximide enabled us to map in detail the cellular response in different parts of the cell cycle. Two aspects of cellular response were

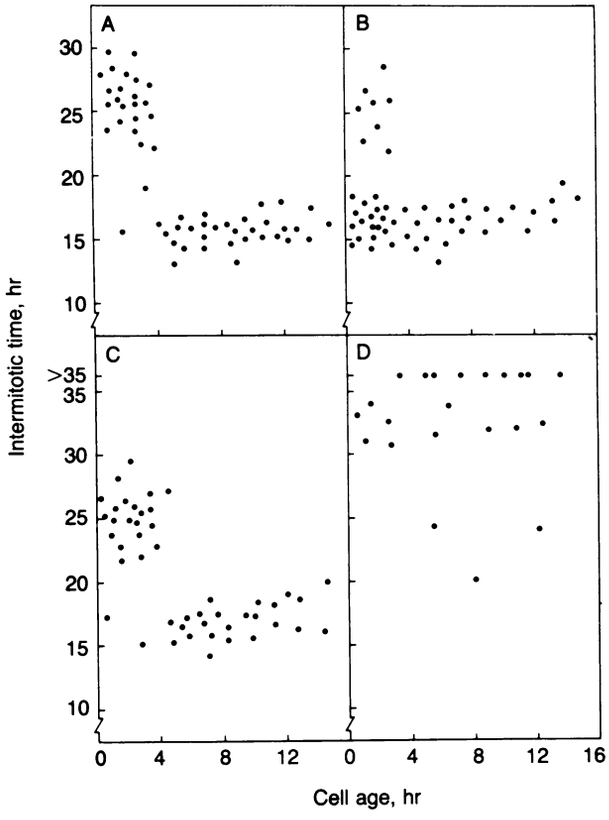

FIG. 5. Relation between cell age at addition of cycloheximide and intermitotic time. Exponentially growing cells at a density of 5000 cells per cm^2 were exposed for 2 hr to serum-free medium (*A*) or to serum-containing medium supplemented with cycloheximide at 10 (*B*), 100 (*C*), or 1000 ng/ml (*D*). Thereafter they were rinsed and exposed for up to 48 hr to cycloheximide-free medium containing 10% FCS. The cell age at the time of cycloheximide addition as well as the intermitotic time for each cell were determined by time-lapse cinematography.

studied and compared: response by arrest in the cell cycle and response by decreased protein synthesis.

Removal of serum was found to have a rapid effect both on protein synthesis and on the ability of the cells to progress through the cell cycle. Inhibition of protein synthesis was observed after 30–60 min in serum-free medium, although maximal inhibition (≈50%) did not occur until 1 hr later. Protein synthesis usually was restored within 30–60 min of readdition of serum. The effect of serum starvation on the ability of cells to progress through the cell cycle was also rapid. Exposure to serum-free medium for only 15–30 min was sufficient to arrest cells in the cell cycle for several hours. In general, however, at least 1 hr in serum-free medium was required for a maximal number of cells to respond. Although protein synthesis was equally depressed in all cell cycle stages after a brief serum starvation, the cellular progression through the cycle was affected in a highly stage-specific manner. Only postmitotic cells younger than 3–4 hr, as defined by time elapsed since previous mitosis, were inhibited in their progression through the rest of the cell cycle. Cells older than 4 hr, on the other hand, were able to traverse the remaining part of the cell cycle with undiminished speed in the absence of serum, although protein synthesis was reduced by 50%.

Transient inhibition of protein synthesis by cycloheximide produced strikingly different effects in different stages of the cell cycle. Although the cells could be blocked in all cell cycle stages if protein synthesis was depressed by ≈90%, only young postmitotic cells were arrested in the cell cycle when

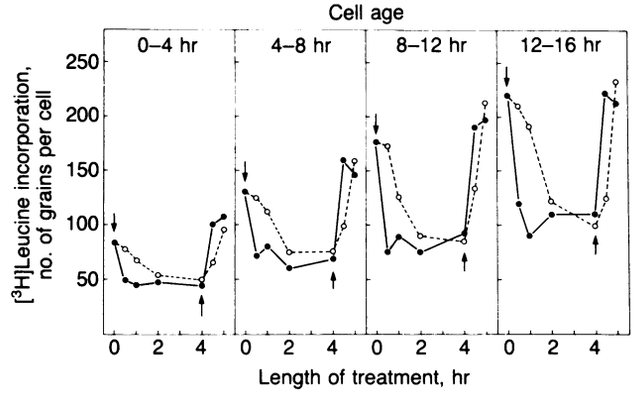

FIG. 6. The relationship between *de novo* protein synthesis and cell age during exposure to serum-free medium or to cycloheximide. Cells exponentially growing on glass coverslips and that had been classified with respect to age (determined by time-lapse cinematography) were exposed either to serum-free medium (○) or to cycloheximide at 100 ng/ml (●) for 0, 0.5, 1, 2 or 4 hr. In two separate experiments, cells that had been exposed to serum-free medium or to cycloheximide for 4 hr were incubated in DME medium containing 10% FCS for 30 or 60 min before protein synthesis was assayed. At the termination of each experimental period, each culture was incubated with 20 μCi of [^{3}H]leucine/ml of medium for 30 min prior to fixation. All glass slides were processed for autoradiography. Protein synthesis was assayed by counting the number of silver grains covering each age-determined cell. Data were pooled for four cell-age groups (0–4, 4–8, 8–12, and 12–16 hr). The arrows indicate addition (↓) and removal (↑) of serum-free medium or cycloheximide. Each point is based on grain counts for 10–20 cells.

protein synthesis was only moderately reduced. Results identical to those seen after brief serum starvation could be produced merely by reducing the level of protein synthesis transiently to 50% by brief exposure to the appropriate dose of cycloheximide. Most of the postmitotic cells younger than 3–4 hr were arrested in the cell cycle, whereas almost all of the cells older than 4 hr progressed to mitosis at an unaltered rate. Even as little as 25% inhibition of protein synthesis arrested many young postmitotic cells, indicating that the progression of these cells through the first 3–4 hr of G_1 is

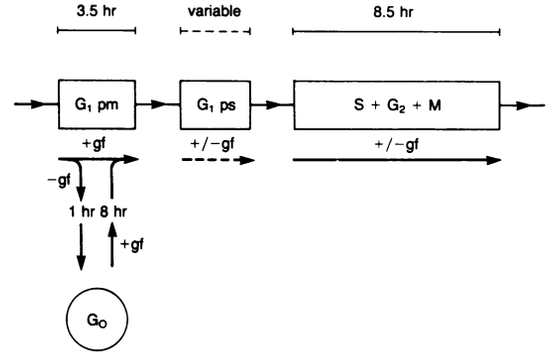

FIG. 7. Schematic model of cell cycle in Swiss 3T3 cells. During the first 3.5-hr after mitosis (G_1pm), the cell makes the decision whether or not to progress through the cell cycle. This decision depends on the presence of growth factors (gf). If the cell senses a lack of growth factors (−gf) in G_1pm, it will leave the cell cycle within 15–60 min and enter a state of quiescence (G_0) from which it takes 8 hr to reenter the cycle after the growth factor level in the environment again becomes optimal (+gf) for proliferation. Once the cell has entered G_1ps, it will eventually initiate DNA synthesis. However, G_1ps is highly variable in length and in fact responsible for most of the variability in the duration of G_1 and of the whole cell cycle.

strongly dependent on a high level of protein synthesis. These findings are consistent with and give further support to the idea that the synthesis and accumulation of one or more labile proteins in G_1 is involved in the major regulatory events leading to proliferation (23–25).

On the basis of the cell-cycle arrest rapidly induced by brief serum starvation or transient inhibition of protein synthesis, the G_1 period of exponentially proliferating 3T3 cells can be subdivided into two physiologically discrete phases: one postmitotic (G_1pm) and one pre-DNA-synthetic (G_1ps). Fig. 7 is a schematic representation of the cell cycle in Swiss 3T3 cells. It is during G_1pm that the cell senses the environment and makes the decision to divide. The entry into G_1ps thus represents commitment for mitosis, and in that sense G_1ps corresponds to G_1c in the terminology of Temin (1), the B state in the terms of Smith and Martin (26), or the part of G_1 after the restriction point according to Pardee (2, 5). However, the approach used in the present study, combining time-lapse cinematographic analysis with brief periods of serum starvation or cycloheximide treatment, revealed new and interesting properties of the commitment process in G_1 and its relation to initiation of DNA synthesis. During optimal culture conditions that support exponential growth, the metabolic events in G_1pm leading to commitment for proliferation are completed at a remarkably constant time after mitosis with little intercellular variability. Most cells leave G_1pm and enter G_1ps in a short time interval between the third and fourth hour after mitosis. This suggests that the growth-regulatory mechanisms in G_1pm—although most likely involving the synthesis of labile proteins as discussed above—are also related to some event or events initiated at or immediately after mitosis, such as changes in the organization of the cytoskeleton (as the cell is changing shape at and after mitosis) or changes in chromatin conformation.

The relation between G_1pm and G_0 was also revealed by the time-lapse analysis. Exposure to serum-free medium or cycloheximide arrested the G_1pm cells for a period that was always found to exceed the actual time of treatment by 8 hr (additional intermitotic delay). This finding is in line with data from Campisi *et al.* (6) showing that BALB/c 3T3 cells were on the average arrested in G_1 for 5 hr after a 3-hr exposure to serum-free medium. The intermitotic delay of 8 hr found in the present study most likely reflects escape from G_1pm to G_0, in which the cells remain during the treatment and from which they require 8 hr to reenter G_1pm upon termination of treatment (Fig. 7). The commitment process in G_1pm is thus not only temporarily stopped as a result of the treatment but more likely rapidly "broken down" to some "idling level" from which it has to start all over again when optimal culture conditions are restored. Although 1 hr of serum starvation is sufficient to force most of the Swiss 3T3 cells in G_1pm into G_0, some G_1pm cells were found to have entered G_0 within 15–30 min after serum removal. Since these rapidly responding cells were evenly distributed over the whole 3–4 hr postmitotic interval, it seems as if the commitment process is equally sensitive to disruption throughout the whole length of G_1pm.

Of particular interest also was the finding that DNA synthesis was not initiated at an invariant time after the completion of the commitment process in G_1, as suggested by previous models (1, 2, 5, 26). As clearly revealed by the time-lapse approach, the time spent in G_1ps could vary considerably from cell to cell. While some cells started DNA synthesis more or less immediately after entering G_1ps, other cells remained in G_1ps for 10 hr or longer. In fact, most of theG_1 variability as well as most of the generation time variability could be ascribed to the observed G_1ps variability. Although G_1ps variability is consistent with a transition-probability event (26, 27), it could also reflect variability in cell size analogous to what has been described earlier for L cells (28, 29).

Time-lapse analysis of cells exposed to purified growth factors during the brief periods of serum starvation revealed differences in cellular response between EGF, insulin, and PDGF. Although all three growth factors individually could counteract the effect of a 2-hr serum-starvation period, only PDGF was able to fully counteract the effect of an 8-hr starvation period. This means that EGF and insulin are insufficient for the final completion of the commitment process in G_1pm, although they can temporarily counteract the entrance of the G_1pm cells into G_0. PDGF, on the other hand, is alone sufficient for successful completion of the commitment process during G_1pm and the subsequent traversal of the rest of the cell cycle in Swiss 3T3 cells.

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